REMARKS

Reconsideration is requested.

Claims 1-62 have been canceled, without prejudice.

Claims 63-91 have been added and are pending. Support for the amended claims may be found throughout the specification and previously pending claims. No new matter has been added.

The interview with the Examiners on April 28, 2004, is acknowledged, with appreciation. The summary of the interview provided by the Examiner in the Interview Summary is an accurate brief description of the issues discussed.

As discussed during the interview with the Examiners, the above-identified application provides, for the first time, an antigenic composition which includes fungal or yeast cell components which are substantially non-proteinaceous (i.e., the antigenic source is characterized by a reduction of antigenic activity of less than 20%, as measured by ELISA, after treatment with protease in 0.25 M TRIS buffer at pH 7.2). As discussed with the Examiners, antigens of the cited art are described as being proteinaceous, as further detailed below. Moreover, one of ordinary skill in the art will appreciate that proteinaceous antigens will be rendered substantially non-antigenic by treatment with protease. Further, one of ordinary skill in the art will appreciate that proteinaceous antigens may generally be frozen or lyophilized without substantial loss of antigenicity.

In characterizing the antigen-containing compositions of present invention, the applicants have described the physical properties of the claimed compositions as being generally resistant to proteases (i.e., reduction of antigenic activity of less than 20%, as

measured by ELISA, after treatment with protease in 0.25 M TRIS at pH 7.2 (see, page 16, last paragraph of the specification, for example) and sensitive to freezing (see, page 8, last paragraph of the specification, for example).

Claim 63, for example, includes a characterization of the physical properties of the antigens of the invention by reference to a test method, i.e., ELISA after treatment with protease under the given conditions. As explained to the Examiners during the interview, claim 63, and now-canceled claim 30, provide a composition. The recitation of a reference method in claim 63, and now-canceled claim 30, does not make these claims product-by-process (i.e., products defined by their method of manufacture) or indefinite, as was believed to be the Examiners positions during the interview, but rather the recitation of the reference method is similar to the physical characterization of the molecular weight of a protein by reference to measurement by a method of electrophoresis, such as by an SDS-PAGE technique.

The Section 102 rejection of claims 30-36, 40-41, 45-46 and 56-57 over Pasarell is moot in view of the above. The pending claims are patentable over Pasarell as, for example, Pasarell prepared their antigen composition by concentration with a PM10 filter. Such a procedure will be recognized by one of ordinary skill in the art to have discarded any material in the culture filtrate which was less than 10,0000 m.w., while retaining larger material, such as antigenic proteins. Pasarell does not literally identify the chemical nature of the antigenic material. One of ordinary skill in the present art, at the time Pasarell was published, would have expected that the antigenic material of Pasarell's filtrate was larger (i.e., > 10,000 m.w.) proteins, which is why the concentration of the antigen-containing filtrate with a PM10 filter was not expected to

alter the antigenic character of the filtrate. As Pasarell fails to literally or inherently teach an antigenic composition characterized by a reduction of antigenic activity of less than 20%, as measured by ELISA, after treatment with protease in 0.25 M TRIS buffer at pH 7.2 (i.e., an antigenic composition which is substantially not proteinaceous), the invention of, for example, claim 63 is submitted to be patentable over Pasarell. The invention of, for example, claim 65, is similarly patentable over Pasarell. As evidenced by, for example, Brewer et al (Can J. Microbiol. 24:1082-1086 (1978)) fungal and yeast toxins, such as aflatoxins, are expected to be non-proteinaceous and of a molecular weight which would be excluded from the antigenic composition of Pasarell by, for example, the PM10 filter used by Pasarell in preparation of their antigenic composition.

For completeness, the applicant notes the Examiner's comment at page 5 of the Office Action dated November 18, 2003 (Paper No. 16) that "There is no requirement in the claims that requires that the cell culture supernatant be filtered with a specific filter." The Examiner's comment however is not understood as it is the cited art (i.e., Pasarell) which requires a specific filter and it is the presently claimed invention which rather does not require a specific filtration step. The invention of claim 63, for example, provides a cell culture supernatant containing fungal or yeast components shed into the supernatant during culturing. The filtration step of Pasarell has removed many of the fungal or yeast components shed into the supernatant during culturing.

The Section 102 rejection of claims 30-35, 37-39, 41 and 45-46 over Takesako (U.S. Patent No. 6,333,164) is moot in view of the above amendments. The pending claim are submitted to be patentable over Takesako and consideration of the following in this regard is requested.

The applicant has previously submitted that Takesako provides protein fungal antigens. In fact, in reviewing the art, Takesako teaches that allergens of fungi exist as a "plurality of antigenic proteins." See, column 4, lines 55-59 of Takesako.[1] Takesako continues in criticizing prior protein and possibly glucan-containing antigen compositions as being undefined and unsatisfactory. See, column 5, lines 1-45. Takesako concludes that "presently available allergen extracts from fungi are completely unsatisfactory. Moreover, there are no known diagnostic and/or therapeutic pharmaceutical compositions in which a sufficient amount of an effective antigen is contained." See, column 5, lines 45-50 of Takesako.

Takesako defines their invention as containing components of cytoplasmic membrane proteins and membrane proteins of cell organelles, obtained from protoplasts derived from fungi causative for infectious diseases," see, column 6, line 67 through column 7, line 3. Takesako provides intercellular cytoplasmic and organelle membrane protein antigens.

The Examiner has responded by asserting that the applicant is "arguing limitations that are not in the claims." See, page 6 of Paper No. 16. As explained above however and to the Examiners during the interview, the recitation of claim 63 that the antigenic activity of the claimed composition is not substantially degraded by protease is a description of a physical and/or chemical property of the claimed invention which distinguishes over Takesako. Moreover, the recitation of claims 63 and 65, for example, that require the presence of fungal or yeast components shed into the

[1] Takesako therefore supports the applicant's position expressed above that one of ordinary skill in the art would believe that Pasarell teaches, at best, a proteinaceous antigenic composition.

supernatant during culturing is contrary to Takesako, as quoted and explained above. Further, the recitation of claim 65 requiring the presence of aflatoxin shed into the supernatant of the culture is contrary to Takesako's composition which contains cytoplasmic membrane proteins and membrane proteins of cell organelles, preferably in a purified and well characterized composition. See, column 12, lines 43-44 of Takesako.

The claims are submitted to be patentable over Takesako.

For completeness, the applicant notes that the fungal antigens of Takesako specifically identified by the Examiner, i.e., Example 19 of Takesako (see, page 7 of Paper No. 16) are protein derivatives of intercellular suspensions of *Aspergillus* (i.e., Af-LSP, Example 2) and *Cryptococcus* (i.e., Crn-LSP, Example 3). The protein antigens of Example 19 of Takesako would be expected to have a substantial loss in antigenic activity if treated with protease.

The Section 102 rejection of claims 30-36, 41-42, 44-51, 55-56, 58 and 62 over van der Heide (Allergy 1985, 40, 592-598) is moot in view of the above. The claims are submitted to be patentable over van der Heide and consideration of the following in this regard is requested.

As discussed during the interview, the cited van der Heide reference refers to an "accompanying paper (6) [Heide, S van der, et al., Allergy 40, 586-591, 1985 (copy attached)] for an explanation of how the "antigens" of the cited reference were prepared. See, van der Heide et al Allergy, 1985, p 593, left column, first full paragraph.

As noted in the attached "accompanying paper", van der Heide's "metabolic antigens" were prepared from a culture media filtrate which was filtered through a 0.2

μm filter and concentrated by hollow fiber filtration (similar to Pasarell discussed above), then dialyzed against tap water and freeze dried. The applicant has demonstrated however that the presently claimed invention is deteriorated by freezing and, more importantly, one of ordinary skill in the art would expect the preparation of van der Heide to be substantially protein in character. The "metabolic antigens" preparation of van der Heide is similar therefore to the prior art described by Takesako and discussed above. Such substantially proteinaceous materials would be expected to substantially loose antigenic activity when treated with protease.

For completeness, the applicant notes that the presently claimed composition which contains antigens is useful, in one respect, for the detection of anti-aflatoxin antibodies. The Examiner's comments relating to any inherent use of Aspergillus antigens of the cited art for detecting aflatoxins *per se* (see, page 8 of Paper No. 16) is not understood. Clarification is requested.

Moreover, the relevance of the Examiner's statement that "aflatoxins are obtained from microorganisms of the genera Aspergillus" is not understood and clarification is requested to the extent the Examiner is asserting that van der Heide inherently provides an antigen composition containing aflatoxin. To the contrary, one of ordinary skill in the art will appreciate that the filtration and freeze drying steps of van der Heide's process for preparing a "metabolic antigen" composition would more than likely produce an aflatoxin-free composition or at least a composition devoid of aflatoxin antigen. The Examiner will appreciate that inherency requires that the allegedly inherent subject matter must necessarily flow from the teaching of the art. The cited art

in the present case however, such as Takesako, provide substantial evidence that the composition of van der Heide would not meet the requirements of the pending claims.

As for the Examiner's assertions that recitations relating to the manner in which supernatant is prepared is an alleged "design choice", and hence perhaps not being considered by the Examiner for purposes of patentability (see, page 8 of Paper No. 16), the applicant urges the Examiner to appreciate that these further process recitations define the physical characteristics of the claimed composition. The applicant has demonstrated that freezing has deleterious effects on the antigenic activity of the claimed invention. The lack of freezing certainly was not an obvious design choice, especially in view of, for example, van der Heide, which describes freeze-drying both the "metabolic antigens" and antigens prepared from mycelium. See, attached van der Heide "accompanying paper".

Finally, for completeness, the applicant notes the Examiner's repeated indication that "Since the [Patent] Office does not have the facilities for examining and comparing applicant's fungal culture supernatant with the fungal culture supernatant of the prior art, the burden is on the applicant to show a novel or unobvious difference between the claimed products and the products of the prior art." See, page 8 of Paper No. 16, and similar comments on pages 3, 6, 10, 13 and 15 of Paper No. 16. The applicant submits that any burden which may have been properly shifted to the applicant has been overcome by the clear indications in the art that supernatant antigens of the cited art were proteinaceous in nature and therefore would have been expected to have suffered a substantial loss in antigen activity if treated with protease. Similar arguments apply to the references discussed below.

The claimed invention is patentable over van der Heide.

The Section 102 rejection of claim 43 over Pasarell stated on pages 10-12 of Paper No. 16 is moot in view of the above. The claims are submitted to be patentable over Pasarell for the reasons noted above.

The Section 112, second paragraph, rejection of claim 54 is moot in view of the above. The undersigned believes that the Examiner indicated during the interview that the Examiner believed there was no support for the subject matter of now-canceled claim 54, as opposed to the claim being indefinite. The Examiner is requested to advise the undersigned if the undersigned misunderstood the Examiner in this respect.

As noted during the interview, support for now-canceled claim 54, as well as pending claim 82, may be found, for example, at page 15, first full paragraph, of the specification. The applicant describes in this paragraph, the surprising and unexpected loss of specificity of the Cladosporium supernatant in carbonate buffer (i.e., under alkaline conditions). As also discussed with the Examiners during the interview, these findings are especially important as the applicant believes that most ELISA testing is performed in alkaline conditions, which are believed to favor adherence of the protein antigens (or proteins in general) to the polycarbonate (or other plastic) ELISA plates normally used. The claims, such as claim 82, is clearly supported by the specification.

The Section 102 rejection of Malling (Allergy, January 41 (1): 57-67), is moot in view of the above. The claims, such as claim 82, are patentable over Malling and consideration of the following in this regard is requested.

Malling teachs evaluation by a skin prick test (SPT), a radioallergosorbent test (RAST), a histamine release (HIST) and a crossed radio immuno electrophonesis

(CRIE). The undersigned believes the Examiner agreed during the interview that of these four evaluation assays, the RAST of Malling is closest to the evaluation recited in now-canceled claim 54, and pending claim 82 (i.e., reacting the antigen composition with sera and determining the serum antibody level). Both this criteria of the claims and the RAST of Malling are immunosorbent assays.

The supernatant of claim 82, and now-canceled claim 54, gives false positive results when used in alkaline conditions. Malling however reports that the "RAST was positive in all clinically positive patients (0/11 = 0% "false positives")". See, page 61, sentence spanning right and left-hand columns. The pH of the RAST procedure is not indicated. In any event, Malling provides no literal or inherent teaching of false positives in immunosorbent assays. The Examiner is requested to clarify her assertion to the contrary at page 13 of Paper No. 16 in the event any of the claims, such as claim 82, is rejected over Malling.

The claims are submitted to be patentable over Malling.

The Section 102 rejection of claims 52 and 53 over Brewer (Canadian Journal of Microbiology, September 1978, 24(9), 1078-81) is moot in view of the above. The claims, such as now-canceled claims 52 and 53, and claims 79 and 80, are submitted to be patentable over Brewer and consideration of the following in this regard is requested.

Brewer teaches isolation of toxic metabolites from mycelia macerated with ice, lyoplylized, macerated with benzene, filtered and extracted three times with benzene. See, page 1083, right column, first full paragraph. The extracts were combined solvent evaporated, and extracted in a methanol layer against petroleum ether and water. Id. Brewer does not teach or suggest an antigenic composition comprising a cell culture

supernatant of the presently claimed invention. The claims are submitted to be patentable over Brewer.

The claims are submitted to be in condition for allowance and a Notice to that effect is requested. The Examiner is requested to contact the undersigned in the event anything further is required in this regard.

Respectfully submitted,

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Allergy, 1985, 40, 586-591

Cultivation of Fungi in Synthetic and Semi-Synthetic Liquid Medium

I. Growth Characteristics of the Fungi and Biochemical Properties of the Isolated Antigenic Material

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Four allergologically important fungi, viz. *Aspergillus fumigatus, Alternaria alternata, Penicillium notatum,* and *Cladosporium herbarum*, were cultured in a pure synthetic medium and the patterns of growth as characterized by the pH, protein and carbohydrate concentration of the culture fluid, were studied. *A. fumigatus* and *P. notatum* showed a similar growth pattern, characterized by a rapid decrease in the pH of the culture medium (pH 7.4→4.0), while proteins were slowly released and saccharose poorly consumed. In contrast, *A. alternata* and *C. herbarum* demonstrated a different pattern of growth, in which the pH of the culture hardly changed during incubation. Enrichment of the synthetic medium with yeast extract greatly improved the growth of all four fungi, as was confirmed by the enhanced yield of antigenic material and strongly increased consumption of saccharose. The yeast extract especially changed the growth pattern of *A. fumigatus* and *P. notatum*, which now is characterized by three phases. Phase I: fall in pH of the growth medium and excretion of proteins; phase II: increase in pH and fall in protein concentration; phase III: stabilization of pH at alkaline values and renewed excretions of proteins. It is concluded that during cultivation of the fungi, the metabolic state of the culture changes, influencing the antigenic composition of the extracts obtained after different periods of cultivation.

Key words: antigens; culture; extraction; fungi; mycelium.

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For many years it has been known that airborne fungi can play an important role in producing respiratory allergy (1, 12). Especially the fungi which belong to the Class of Deuteromyceten or Fungi Imperfecti are a potential source of allergenic material. Fungal extracts which are used for diagnostic purposes vary strongly in allergenic potency and allergen content (13, 14, 18). One reason for this variability in potency is the variability in strains used; differences in antigenic composition between different strains have been reported (9, 15, 16). Another important contribution to the variability in antigenic potency of antigenic extracts may be differences in cultivation procedures (surface of submerged cultures), and the origin of the fungal material used for extraction, such as spores, mycelium or culture fluid (4, 7, 17). Information about the relation between antigenic or allergenic composition of fungal extracts and growth characteristics during cultivation is essential for improving the standardization of fungal extracts. Knowledge of these characteristics, however, is limited (2, 5, 7, 19). Within the framework of allergen standardization we therefore studied the growth characteristics of four allergologically important fungi, viz. *Aspergillus fumigatus, Alternaria alternata, Penicillium notatum,* and *Cladosporium herbarum* and the corresponding IgG- and IgE-binding properties of the isolated antigenic and allergenic fractions.

In this study, the growth characteristics of

these four fungi are described, using pH, protein and carbohydrate concentrations of the culture fluid as parameters of growth.

Since cultivation of these fungi in pure synthetic medium was impaired compared with an organic medium (Sabouraud), the effect of the addition of small amounts of yeast extract to the culture medium on the growth characteristics was also studied.

MATERIAL AND METHODS

Fungal strains: *A. alternata* (CBS 104.26), *P. notatum* (CBS 205.57), *C. herbarum* (CBS 366.80), were obtained from the Centraal Bureau voor Schimmelcultures, Baarn, The Netherlands (Dr. G. A. de Vries).

The *A. fumigatus* strain was isolated from the sputum of a patient with an aspergilloma; this strain has been routinely used in our laboratory for preparing antigenic extracts (5, 6).

Cultivation of fungi

The fungi were grown in 2 % malt agar slants at 27° C until sufficient sporulation occurred. An Erlenmeyer flask (500 ml) containing 150 ml of Czapek Dox medium (Difco Laboratories) was inoculated with 0.1 ml of a spore suspension, containing about 1000 spores in 0.5 % Tween 20. After 3 days of incubation in a rotatory shaker (New Brunswick) at 120 rpm and 27° C, about 10 fungal balls of the exponential growing cultures were transfered to Erlenmeyer flasks of 2 l containing 1 l of Czapek Dox medium (with or without the addition of 0.1 % yeast extract). At regular time intervals during cultivation at 27° C and 120 rpm, samples were taken for measuring the pH, protein and carbohydrate concentration in the culture fluid.

Preparation of antigens

After 10 and 28 days of cultivation, the mycelium was harvested by Buchner filtration. The culture medium filtrate was filtered again with a Seitz filter (0.2 µm) and concentrated by H,P, hollow fiber filtration (Amicon). After dialysation against streaming tap water the dialysate was freeze-dried. The mycelium mat on the filter was washed with deionized water and stored at -20° C.

Mycelial antigens were prepared as described by Kim & Chaparas (7). Briefly, the thawed mycelium was supended in distilled water and homogenized with an Ultra Turrax blender. The mixture was then homogenized in a Braun mechanical cell homogenizer for a few minutes, using 0.45 µm glass beads and intermittent chilling with CO_2. Complete disruption of cells was checked by microscopic examination. After centrifugation, the supernatant, filtered with Seitz filter (0.2 µm), was dialysed against streaming tap water and freeze-dried.

Chemical composition

Protein concentration was determined by the method of Lowry et al. (10). Carbohydrate content was estimated by the phenol-sulphuric acid method with a modification of McKelry et al. (11).

RESULTS

The growth characteristics of the four fungi in synthetic medium with and without the addition of yeast extract (YE) are shown in Fig. 1 (a-d). Without the addition of YE, the growth patterns of *A. fumigatus* and *P. notatum* were very similar and characterized by a steep drop in the pH of the culture fluid (Fig. 1a, b, left figures). During further incubation the pH in the medium remained low, a small increase in pH was noticed for *A. fumigatus*. The fall in pH was accompanied by a gradual increase of the protein content, which was more pronounced for *A. fumigatus*. The sugar concentration in the medium decreased slowly. In contrast to *A. fumigatus* and *P. notatum*, the two other fungi, *A. alternata* and *C. herbarum* did not show large changes in the pH of the medium, which remained almost constant during the time of growth (Fig. 1c, d). Also here the sugar concentration in the medium diminished slowly. The growth of the fungi in pure synthetic medium was slower and the yield of antigenic

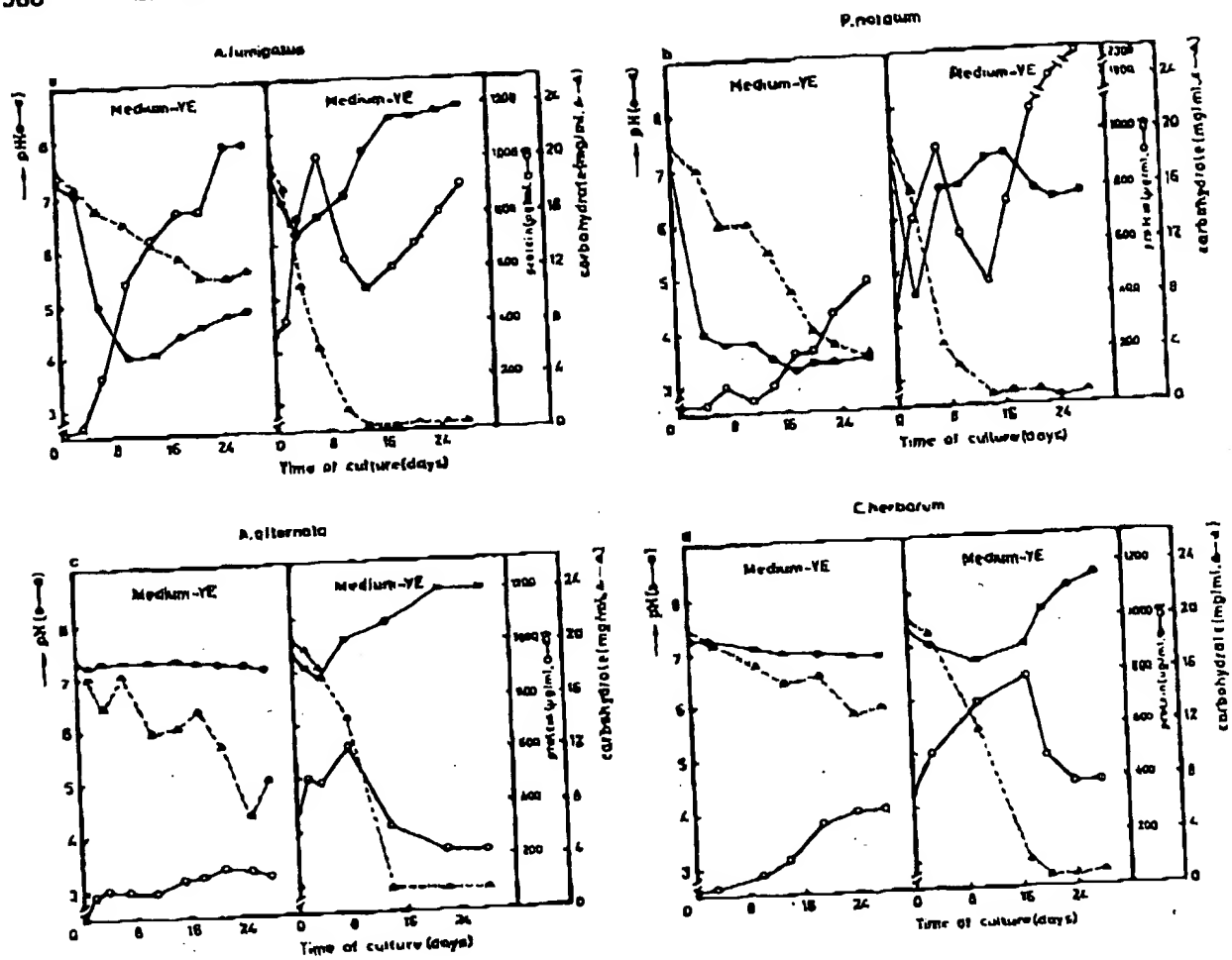

Fig. 1. Growth patterns of the four fungi, characterized by the pH (●——●), protein concentration (○——○), and carbohydrate content (▲------▲) of the culture fluid, from cultures without (–) and with (+) yeast extract (YE) in the culture medium.
a: *A. fumigatus*; b: *P. notatum*; c: *A. alternata*; d: *C. herbarum*.

material was low when compared with growth in a rich Sabouraud medium (5). When *A. fumigatus* was cultured in the presence of different concentrations of YE in 150 ml Czapek Dox medium in 500 ml Erlenmeyer flasks, addition of YE to the medium greatly improved the rate of growth of *A. fumigatus*, as is reflected by the recovery of dry-mycelium and culture fluid antigens (Table 1). Addition of 0.1 % YE strongly enhanced the yield of antigenic material. Therefore, all four fungi were cultured in the presence of 0.1 % YE in the Czapek Dox medium and the growth characteristics are shown in Fig. 1 (right figures).

With YE in the medium, *A. fumigatus* and *P. notatum* demonstrated growth patterns (Fig. 1a, b, right), which are almost identical to those of *A. fumigatus* grown in a Sabouraud medium (5). In the first phase of growth there is a short fall in the pH of the culture fluid. This is accompanied by a rise in the protein concentration, which probably reflects the active excretion of several enzymes. The sugar content in the medium now rapidly declined to zero levels, indicating that the saccharose in the Czapek Dox medium was more rapidly used for fungus growth.

The second phase of growth is characterized by a rise in the pH of the culture fluid and a fall in protein concentration. In this phase the fungal mass is still increasing.

During the third phase of growth the pH of the culture remains almost constant. The protein concentration of the medium increased

during this period, probably due to liberation of intracellular proteins after lysis of the cells (5).

The growth of the other two fungi, *A. alternata* and *C. herbarum*, was also improved by the addition of YE to the medium (Fig. 1c, d, right) but now only two phases of growth can be distinguished, based on a small fall in pH (phase I), followed by a moderate increase to more alkaline values (phase II). During phase I an excretion of proteins was observed while the saccharose was rapidly consumed, comparable with phase I conditions of *A. fumigatus* and *P. notatum*. In phase II reconsumption of proteins was found, whereas saccharose concentration reached zero levels and the pH was increased above initial value.

The observations with *A. alternata* and *C. herbarum*, as described above, are in agreement with the finding that both fungi showed less lysis during the 28 days of cultivation when compared with *A. fumigatus* and *P. notatum*. The faster growth of the four fungi after addition of YE to the Czapek Dox medium is reflected by the enhanced yields of culture fluid antigens and mycelium antigens as is shown in Table 2. In all cases (except for the culture filtrate

Table 1

Effect of different concentrations of yeast extract (YE) in the culture medium on the yield of dry mycelium and culture filtrate (CF)-antigens from *A. fumigatus* (in mg/150 ml culture medium)

	10 d. culture		28 d. culture	
	Mycelium	CF-antigens	Mycelium	CF-antigens
Without YE	187	52	677	72
0.1 % YE	1626	85	960	149
0.25 % YE	1884	87	1044	158
0.5 % YE	2123	98	1380	151

Table 2

Influence of 0.1 % yeast extract (YE) in the culture medium on the amount of antigenic material (in mg dry weight/100 ml culture fluid) from culture filtrate (CF) and mycelium (M) of early phase (e) and late phase (l) cultures

Antigenic extract	Medium without YE	Medium with YE
A. fumigatus		
CF-e	9	19
CF-l	17	40
M-e	14	57
M-l	29	32
A. alternata		
CF-e	9	13
CF-l	15	40
M-e	8	29
M-l	16	33
P. notatum		
CF-e	8	28
CF-l	17	60
M-e	14	20
M-l	23	31
C. herbarum		
CF-e	12	19
CF-l	65	56
M-e	7	40
M-l	22	49

Table 3

Influence of 0.1 % yeast extract (YE) in the growth medium on the protein/carbohydrate ratio (P/C) of antigenic extracts, obtained from early phase (e) and late phase (l) cultures. CF: antigens from culture fluid; M: mycelial antigens

Antigenic extract	P/C ratio	
	Medium without YE	Medium with YE
A. fumigatus		
CF-e	0.50	0.38
CF-l	0.58	0.46
M-e	0.44	9.28
M-l	1.72	1.37
A. alternata		
CF-e	0.40	0.30
CF-l	0.36	0.33
M-e	n.d.	0.64
M-l	0.85	1.50
P. notatum		
CF-e	1.36	0.46
CF-l	0.45	0.92
M-e	1.12	0.80
M-l	1.00	1.54
C. herbarum		
CF-e	0.10	0.14
CF-l	0.13	0.30
M-e	0.25	0.37
M-l	0.21	0.58

n.d.: not determined.

antigen from *C. herbarum* after 28 days of growth) more antigenic material has been isolated from cultures, after the addition of YE to the medium.

Yeast extract itself did not contribute to the enhanced recovery of antigenic material because it consists largely of small molecular weight compounds, which are mainly metabolized during growth of the fungi while residual material is lost during dialysation. In addition, more antigens were obtained from 28-day cultures (1) than from 10-day cultures (e). Chemical analysis of the different antigenic extracts showed that the protein/carbohydrate ratios (P/C) of the antigenic extracts hardly changed after addition of YE to the medium (Table 3). The P/C ratios were similar to those reported for *A. fumigatus* by Kim & Chaparas (7) and Wallenbeck et al. (16).

In general, the mycelial antigens from late phase cultures showed the highest P/C ratio, which was independent of YE in the culture medium. An exception is the early phase mycelial antigen of *A. fumigatus* from a culture with YE in the medium which showed a much higher P/C value (3.28) when compared with other values.

DISCUSSION

For standardization of fungal allergenic extracts more knowledge about culture conditions, metabolic states of the microorganisms, and corresponding antigenic/allergenic composition is necessary. In this study growth characteristics of four allergologically important fungi, viz. *A. fumigatus*, *A. alternata*, *P. notatum*, and *C. herbarum*, were investigated. The four fungi do grow in the pure synthetic medium as shown in Fig. 1 (left figures). For *A. fumigatus* and *P. notatum* the growth patterns (as characterized by pH, protein and carbohydrate content of the culture fluid) were similar and changed strongly during time of cultivation. The culture became acidic very rapidly and only a limited increase in pH during further cultivation was observed. In contrast to these fungi, *A. alternata* and *C. herbarum* demonstrated a different growth pattern, in which the pH of the culture hardly changed during cultivation. Changes in metabolic states of the cultures in time were furthermore demonstrated by changes in the protein/carbohydrate ratio of the antigenic extracts of mycelium and culture fluid of early phase (after 10 days of growth) and late phase (after 28 days) cultures (Table 3). It was shown that also the antigenic fractions of *A. alternata* and *C. herbarum*, isolated after different periods of cultivation differed in P/C ratio, whereas the pH of the culture fluid of these two fungi hardly changed.

Because of the relatively impaired growth of the fungi in pure synthetic medium when compared with growth in an organic medium, the synthetic medium was enriched with yeast extract (YE).

Addition of 0.1 % YE to the medium greatly improved the growth of the four fungi, as was confirmed by an increase in the yields of mycelium and culture fluid (antigenic) material excreted by the microorganisms (Table 2). Furthermore, the use of YE in the culture medium had a strong influence on the metabolic state of the cultures, especially on those of *A. fumigatus* and *P. notatum* (Fig. 1a, b). Without YE the cultures remained in an early growth state (acidic state), whereas after addition of YE the full cycle of growth states I–III, as described for *A. fumigatus* in organic medium (5) was passed. It is concluded that YE contains nutrients (vitamins) which are essential for fungal growth and allow the fungi to make better use of saccharose as a carbon source for their growth. Due to the improved growth of the microorganisms, more components are excreted into the culture medium. In this study it was shown that the time of cultivation influences the metabolic state of fungal cultures. Therefore it is plausible that also the antigenic composition of the fungal extracts will depend on the time of cultivation.

The accompanying paper shows the corresponding immunological properties (precipitation pattern and IgG- and IgE-binding capacity) of the extracts described above and the influence of yeast extract in the culture fluid on these parameters (3).

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